보건학석사 학위논문

Disruption of the steroidogenesis and steroid metabolism pathway in C57BL/6 mice after prenatal exposure to bisphenol S

비스페놀 S의 태중 노출로 인한 C57BL/6 마우스 내의 스테로이드 합성 및 대사 경로 교란 연구

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Abstract

Disruption of the steroidogenesis and steroid metabolism pathway in C57BL/6 mice after prenatal exposure to bisphenol S

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Bisphenol S (BPS) is a substitute for bisphenol A (BPA), and widely used in the manufacture of polycarbonate plastics, epoxy resins and consumer products. BPS has been reported as endocrine disruption chemicals (EDCs) that cause weak estrogenic or anti-androgenic activity. Although many studies related to BPS have been conducted, the steroidogenesis disruption of prenatal exposure to BPS on mammals study has not yet been elucidated.

The objective of the study is to identify differences in steroidogenesis and steroid hormones metabolites due to prenatal exposure to BPS by sex and time point. In present study, the C57BL/6 pregnant mice were exposed to BPS 5mg/kg bw/day, 50mg/kg bw/day, 250mg/kg bw/day through drinking water for about 10days (gestation period 9 days – delivery). F1 generation was sacrificed at 8 weeks and 23 weeks of age, and physiological characteristics,

serum, fat, organs were collected. Using the collected serum, six hormones (Gonadotropin releasing hormone (GnRH), Follicle stimulating hormone (FSH), Luteinizing hormone (LH), Progesterone (P4), Testosterone (T), Estradiol (E2)) were measured by ELISA kit. In addition, UPLC-qTOF was used to measure steroid hormones and their metabolites involved in the synthesis and metabolism of steroid hormones in serum. Physiological statistical analysis of general linear model showed a significantly different tendency depending on sex, age and exposure concentration. In particular, both male and female mice group showed significant differences in puberty (8-week-old) than in adulthood (23-week-old). The hormones involved in the HPG axis, in the 250mg/kg bw/day group in 8-week-old female and male group, LH and T values tended to increase as GnRH increased. Among the metabolites related to steroidogenesis and metabolism pathway, 23-week-old male and female group, 8-week-old male group showed a tendency to metabolize from pregenolone to tetrahydrocorticosterone at all dose. This study showed that, compared to BPS in vitro test results, prenatal exposure to BPS cause androgenic activity, unlike a weak estrogenic agonist activity. However, since the steroid hormone synthesis in the adrenal gland cannot be detected, it is difficult to explain the UPLC-qTOF detection results. The results of the study showed that the effects of physiological and hormones on prenatal exposure to BPS were significantly different according to the sex, exposure concentration, time point. Therefore, in present study provides information on changes in the circulation system of steroid hormone synthesis and metabolism caused by exposure to BPS in rodents and could be used for the further research of toxic mechanism of BPS.

Key words : Bisphenol S, prenatal exposure, HPG axis, steroidogenesis and metabolism, androgen activity

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Ⅰ. Introduction

The principles of "Developmental Origins of Health and Disease" (DOHaD) describe how early life exposures in conception, pregnancy, infancy and childhood between maternal and environmental factors can have a significant impact on health and disease risk in later life (Barker, 1990, 1995; Li et al., 2008; Charles et al., 2016). One of the environmental factors is endocrine-disrupting compounds (EDCs). Exposure to EDCs, a group of biologically active compounds that mimic or antagonize the effects of endogenous hormones, during critical stages of differentiation can interfere with the balance of hormones necessary for normal development, and result in altered gene expression that is not detectable until puberty or much later in life (Zoller et al., 2016).

Bisphenol S (BPS;4,4′-sulfonyldiphenol) is composed of two phenol groups on each side of a sulfonyl group. It has been used in the manufacturing of epoxy resins and polycarbonate plastics as constituents of a wide variety of consumer products (Liao et al., 2012a; Simoneau et al., 2011). It has been introduced as a substitute for bisphenol A (BPA), one of the representative endocrine disrupting chemicals, due to the structural analogy and higher thermal stability (Lotti et al., 2011). BPS has also been detected in surface water, sediment, and sewage effluent, generally at lower concentrations than BPA, but in the same order of magnitude (Fromme et al. 2001; Song et al. 2014; Yang et al. 2014). In human, BPS has been detected in urine at concentrations and frequencies comparable to BPA (Liao et al. 2012a; Zhou et al. 2014).

Several studies have demonstrated that BPS shows weak estrogenic activity both *in vivo* and *in vitro*. An in vitro study demonstrated that BPS induced membrane ERα – mediated pathways and actions: MAPK(mitogen-activated

protein kinase) signaling, cell proliferation, and activation of caspase 8 (Viñas and Watson 2013a, 2013b). Another study demonstrated that BPS has androgenic activity similar to BPA (Molina-Molina et al., 2013). A recent study demonstrated that BPS non-monotonically reduced the basal testosterone secretion in human fetal testes and exhibited anti-androgenic effects (Eladak et al., 2015). In vivo study reported that BPS exposure was estrogenic in rats via increases in uterine weight, and also found to bind the estrogen receptor (Yamasaki et al., 2004). A recent study demonstrated that BPS decreases in gonad weight in males and females, and also E2 levels were increased in males and in females in zebrafish (Ji et al., 2013). Although many studies related to BPS have been conducted, the steroidogenesis disruption of BPS on mammals study has not yet been elucidated.

We performed the present study to fill this knowledge gap as follows: endocrine disruption effects of BPS of reproduction-related hormones along hypothalamus-pituitary-gonadal (HPG) axis (Fig.1) were studied. And also steroid hormones and metabolites associated with steroidogenesis and metabolism pathway (Fig.S3) were studied. The objective of the present study is to identify differences in steroidogenesis and steroid hormones metabolism changes due to prenatal exposure to BPS by sex and time point.

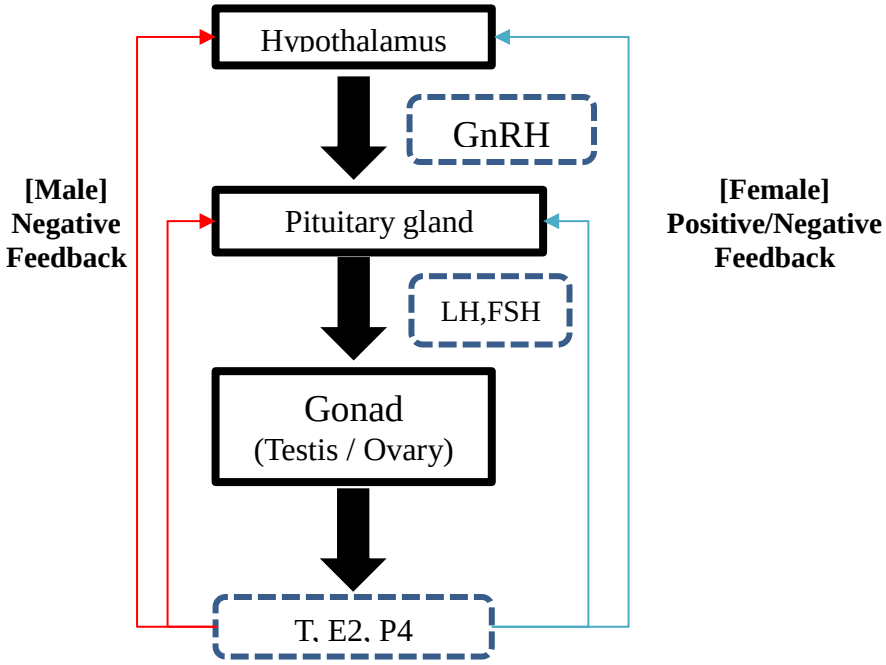


Fig 1. Hormone secretion in the hypothalamus-pituitary-gonadal (HPG). GnRH, Gonadotropin-releasing hormone; LH, Luteinizing hormone; FSH, Follicle-stimulating hormone; E2, Estradiol; T, Testosterone; P4, Progesterone.

II. Materials and methods

2.1. Chemicals

Bisphenol S (BPS, CAS No. 80-09-1, purity 98%) were purchased from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO, U.S.A.). Acetonitrile and Water (HPLC grade) were purchased from Avantor (Center Velly, PA, U.S.A.). And Ethyl alcohol (99.8%, CAS No. 64-17-5), Formic acid (98% pure, CAS No. 64-18-6) were obtained from Thermo Fisher scientific Inc. (Waltham, MS, U.S.A.).

2.2 Animals and treatment

All experimental animals were housed and handled in accordance with Guide-lines and Regulations in Seoul National University. 12 weeks to 14 weeks old C57BL/6 pregnant mouse were used in this study and purchased from Koatech, Korea. C57BL/6 pregnant mouse were maintained under controlled temperature (22.5-25 ℃) and humidity (50-70%) with a 12hr light-dark cycle (06:00-18:00). BPS dissolved in 0.5% ethanol was administrated orally via distilled water in glass bottle to treatment pregnant mouse from gestation day (GD) 9 to until delivery (GD18-19). During the gestation period, 10 pregnant mouse from the control group (Vehicle, V) and 26 pregnant mouse from the treatment group were treated daily at dose of 0 (V), 5 (L), 50 (M), 250 (H) mg BPS/ kg of body weight/ day in 0.5% Ethanol. Since the reference dose (RfD) of BPS has not yet established, the administration concentration refers to the RfD of BPA. The Lowest-observed-adverse-effect-value of BPA was 50mg/

kg bw/ day (U.S. Environmental Protection Agency National Center for Environmental Assessment). We selected 10 times lower concentration and 10 times higher concentration based on NOAEL. On the day of birth (postnatal day (PND) 0), litters born to treated and control dams were divided by sex, time point after weaning day. 5 male, female pups (F1) per cage were housed with distilled water and normal diet. Feed, water and cages were changed once a week. The study was conducted according to protocols approved by the Institutional Animal Care and Use Committee of Seoul National University, Korea (IACUC No. 160930-3-1).

2.3 Measurement of physical parameters and sampling procedures

Body weight of pups were measured every weeks by use of an electronic balance. And Body length and Anogenital distance (AGD) were measured by use of a digital caliper (Bluebird Inc., Korea). Each animal per dose was sacrificed at 8 weeks old and 23 weeks old, respectively. Blood was collected and centrifuged at 15,000 rpm for 15min at room temperature. The serum was then collected and stored at –80℃ until further analysis. All organs were isolated and weighed and then were frozen in liquid nitrogen and stored at –80 ℃.

2.4 ELISA for Gonadotropin-releasing hormone (GnRH) and steroid hormones

All hormones were measured using serum. Luteinizing hormone (LH), Gonadotropin-releasing hormone (GnRH), Follicle-stimulating hormone (FSH), Estradiol (E2), Testosterone (T) and Progesterone (P4) were measured using an enzyme-linked immunosorbent assay (ELISA) kits (LH, Lifespan Biosciences Inc., U.S.A; GnRH, FSH, E2, Cusabio Biotech Co., China; T, P4, Cayman chemical, U.S.A) according to the manufacturer's instruction.

2.5 Sample preparation

All serum samples were thawed at 4°C before pretreatment. A 4μL aliquot of each serum sample was used for metabolite extraction using UPLC-QTOF-MS. Place 40 μL of serum in a sterile screw tube, after addition of 120 μL acetonitrile to serum. The mixture was successively vortex-mixed for 1min, and then incubated at −20°C for 20 min, and centrifuged at 10,000×g for 10 min at 4 °C. Then, the resulted supernatant was evaporated to dryness under N2, and a 40 μL aliquot of ultrapure water was added into each tube to reconstitute the dried sample. Afterwards, the solution was vortex-mixed for 1min, and the supernatant was transferred into a total recovery glass vial (Waters, USA) pending UPLC-MS analysis. All samples were kept at 4°C inside the auto-sampler during the analysis. (Dong et al., 2015)

2.6 UPLC-QTOF-MS analysis

Ultra Performance Liquid Chromatography (UPLC) was performed using a Waters ACQUITY UPLC H-class (Waters Corp., Milford, MA.). A 5 μL aliquot of each sample solution was injected into an ACQUITY BEH C18 column (2.1mm × 100mm, 1.7 μm). The column temperature was set to 30 °C, and the flow rate of mobile phase was 400 μL/min. Analytes were eluted from column with a gradient, where phase A was water with 0.1% formic acid and phase B was acetonitrile with 0.1% formic acid. The initial composition of B was 5%, and increased linearly to 80% in 10min, and then to 100 % in the following 10 min. The mobile phase maintained at 100% B for 5 min and another 5 min at 5% B before the next injection. Each run time was 30 min (Dong et al., 2015).

Next, MS analysis was performed using a Waters Synapt G2-Si MS (Waters Corp., Milford, MA) using an electrospray ionization (ESI) operated in the positive and negative ion mode. The mass spectrometers performed alternative high- and low- energy scans, known as the MS^E acquisition mode. The operating parameters were as follows; cone voltage, 40V; capillary voltage, 5.0kV; source temperature, 120℃; desolvation temperature, 250℃; cone gas flow, 30 L/h; and desolvation gas flow, 600 L/h. The scan mass range was from 100 and 1,000 *m/z*. Data was scanned from m/z 50 to 1000 with a scan time of 0.01 s (Dong et al., 2015).

The QTOF-MS data was collected in continum mode, using the lock spray to ensure accuracy and reproducibility. A concentration of 1ng/mL leucine-enkephalin was used as lock mass (m/z 556.2771 (ESI+), m/z 554.2615 (ESI-)). The lock spray frequency was set at 10s, and the lock mass data were averaged over 10 scans for correction. All of the data acquisition was controlled using Waters MassLynx software (Waters Corp. Milford, MA) with the MS^E program.

2.7 Data processing

All MS^E data were processed within UNIFI software (Waters Corp., Milford, USA). We created the user library of over 90 species of metabolites presented in Fig S1. Based on this user library, All MS^E data peaks were identified through UNIFI. Among the identified peaks compared the mass error, the number of fragments, and the mass error of the fragment to determine a meaningful peak. And then, the three-dimensional data including peak number (RT-m/z pair), sample name, and normalized peak areas were exported to the EZ info software 3.0.3 (UMETRICS) for multivariate analysis. The detected metabolites were compared with the control group to determine the change of metabolic pathway.

2.8 Statistical analysis

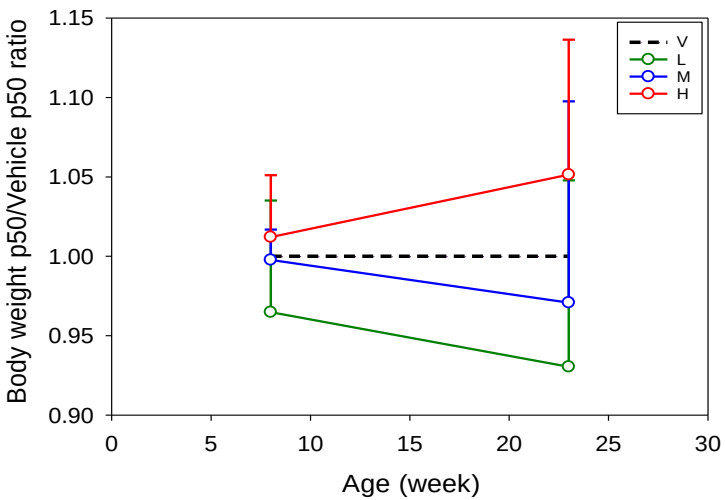
The physiological data were presented as median (p50) divided vehicle median with standard error (p75) ratio. The ELISA kit data were presented as mean with standard deviation (mean $\pm$ SD). Statistical analysis was performed using SAS 3.3 software (SAS Institute Inc., Cary, U.S.A). Kit data were not normally distributed, so these data were transformed natural log (ln), followed by statistical analysis. General linear model analysis (GLM) were performed to compare data among dose of BPS. All p values < 0.05 was considered to be significant.

Ⅲ. Results

3.1 Body weight, Body length, AGD

The physical measurements corresponding to each group are shown in Fig 2, Fig 3, Fig 4. In the graph, the median value of the control group (Vehicle) was set as the reference (Vehicle p50 / Vehicle p50 =1). The median value of each dose was divided by the median value of the control group. Body weight did not show a significant difference, however the tendency was sex difference and dose difference. Body length of the male mice was shorter than that of the control group, while the length of the H group was longer. In female mice, the L group was similar in body length to the control group, and the M and H groups had longer than the control group. Especially, the H group of 8-week-old female mice had significantly longer body length, however there was no significant difference at 23-week-old. AGD (Anogenital distance) was significantly different by sex. In 8-week-male mice, AGD of mice exposed to M (50mg BPS/kg/ day) was significantly longer than that of control mice. AGD of mice exposed to L (5mg BPS/kg/ day) and H (250mg BPS/ kg/ day) concentration was slightly longer than that of the control group. However, in 23-week-male mice AGD of mice exposed to L was shorter than that of AGD of control group, and AGD of mice exposed to H was significantly longer than that of AGD of control group. In female mice, AGD was longer at 8-week-old than control, and decreased at 23-week-old at all exposed group.

(A) Body weight ratio of male mice



(B) Body weight ratio of female mice

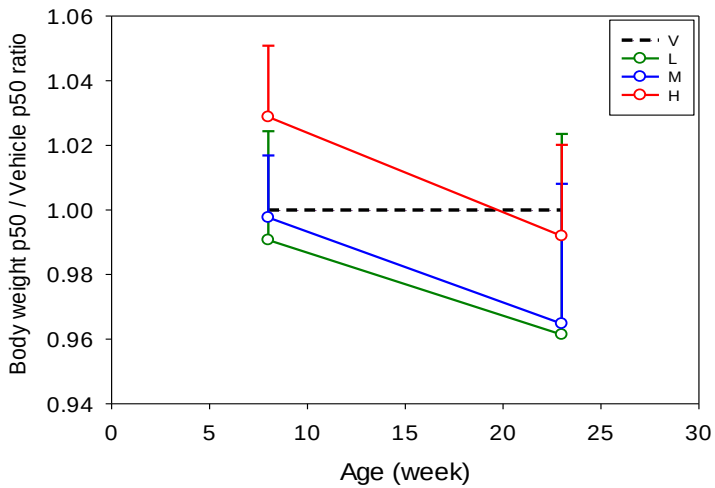
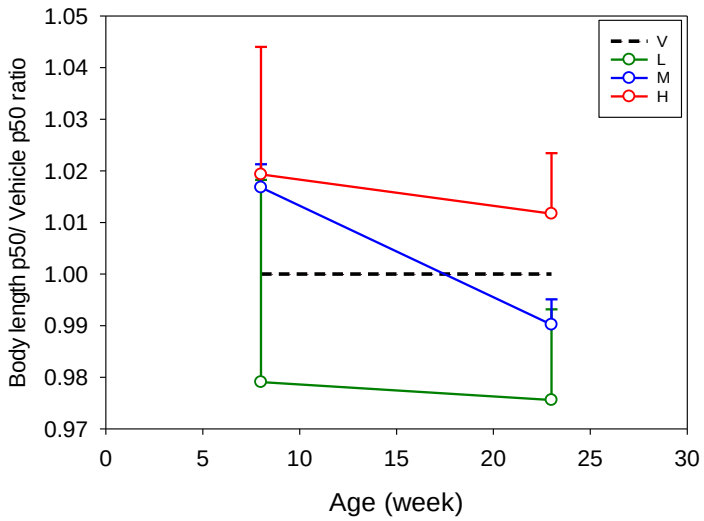


Figure 2. Body weight ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle).

(A) Body length ratio of male mice



(B) Body length ratio of female mice

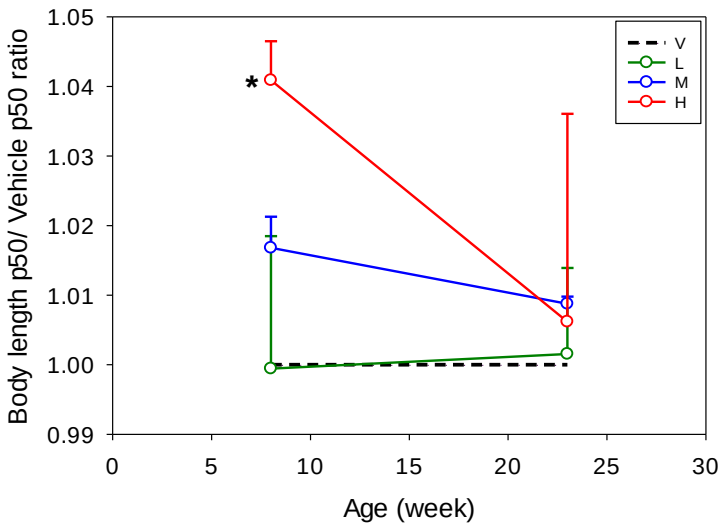
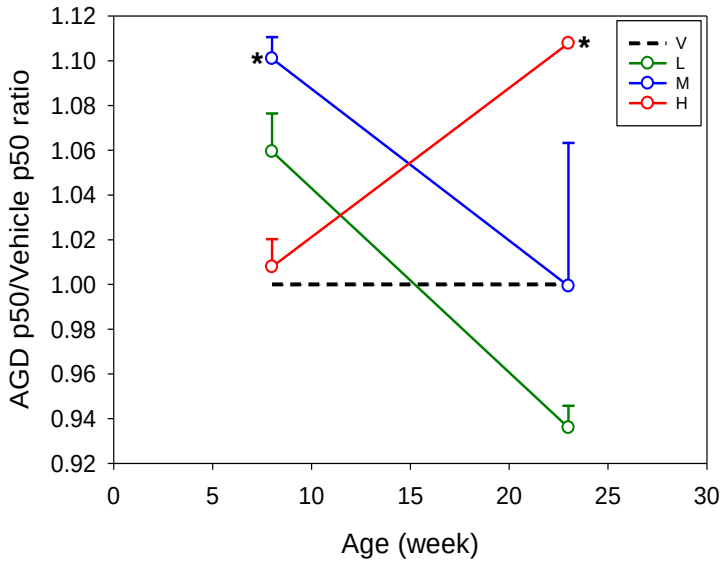


Figure 3. Body length ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle).

(A) AGD ratio of male mice



(B) AGD ratio of female mice

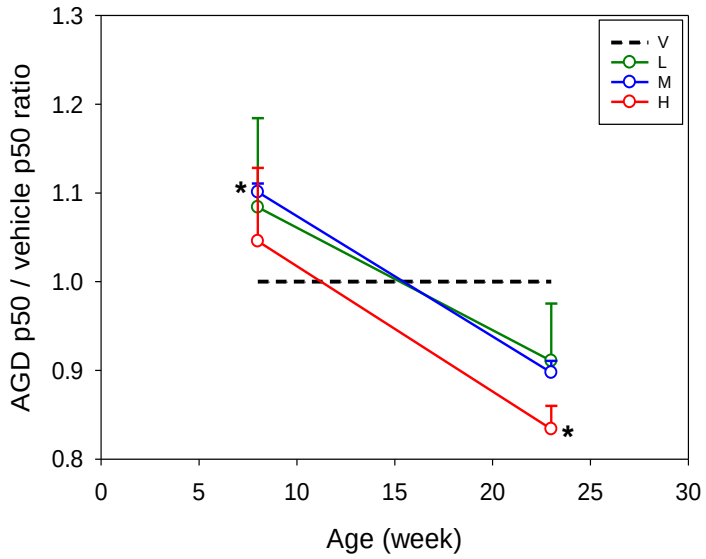


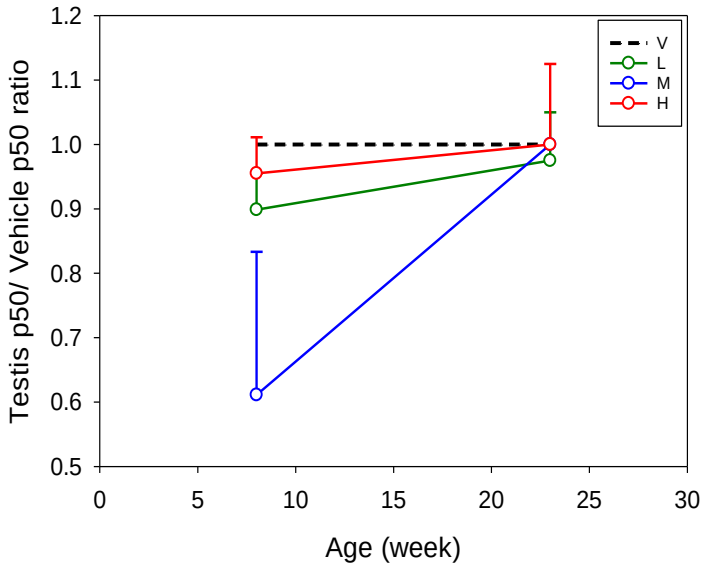
Figure 4. AGD (Anogenital distance) ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle).

3.2 Effect of BPS on organ weights of mice

Fig 5, Fig 6, Fig 7 are shown the organ weights p50 ratio such as gonad (testis in male, ovary in female), liver, kidney adjusted to organ weights median of control group by sex, time point. Each organs ratio were shown the tendency by sex difference and dose difference. Gonad was smaller than control group at all sex, dose at 8-week-old. In particular, the female mice exposed to L (5mg BPS/ kg /day) group were significantly smaller in size. However, at 23-week-old, the gonad size of exposure group was similar to that of the control group. In Liver, any differences did not show by sex, time point, dose. In kidney, there was a significantly differences at 8-week-female mice exposed to L, H group. Growing to 23-week-old female mice was shown a tendency to similar to that of the control group at H group. However, the kidney ratio of L group did not change with age from 8-week-old to 23-week-old by dose.

(A) Testis ratio of male mice



(B) ovary ratio of female mice

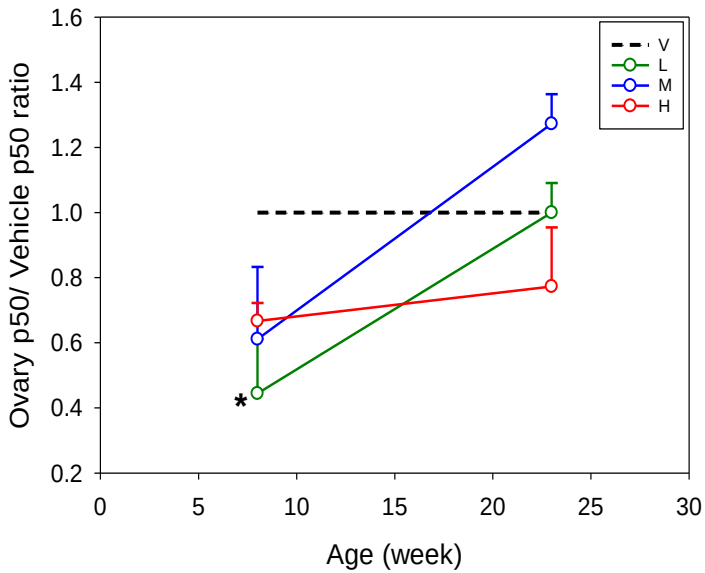
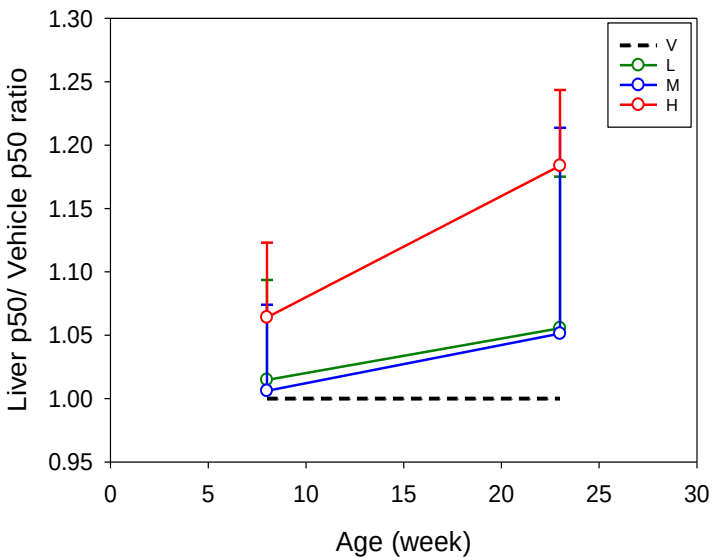


Figure 5. Gonad ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle).

(A) Liver ratio of male mice



(B) Liver ratio of female mice

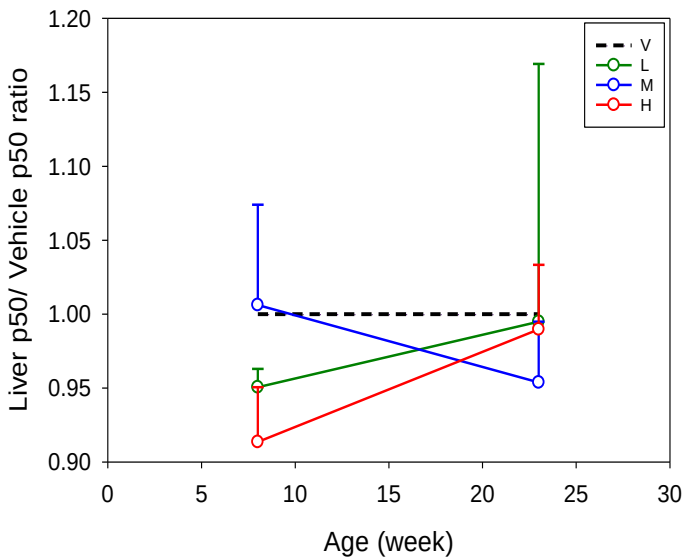


Figure 6. Liver ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle).

(A) Kidney ratio of male mice

(B) Kidney ratio of female mice

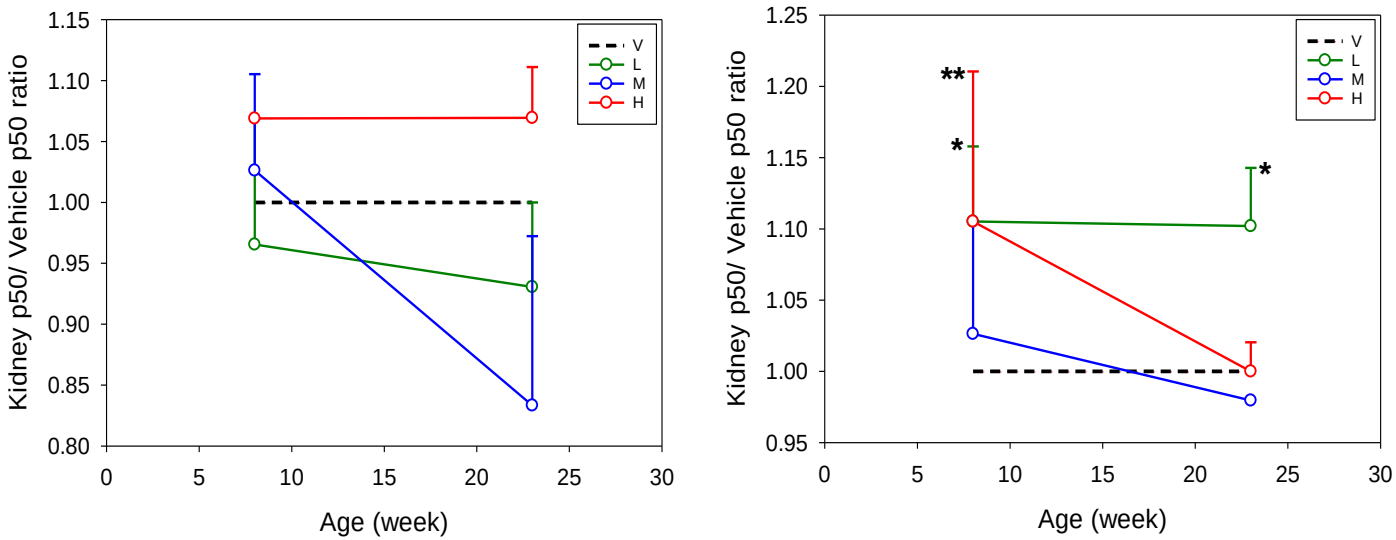


Figure 7. Kidney ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle).

3.3 Effect of BPS on fat tissue of mice

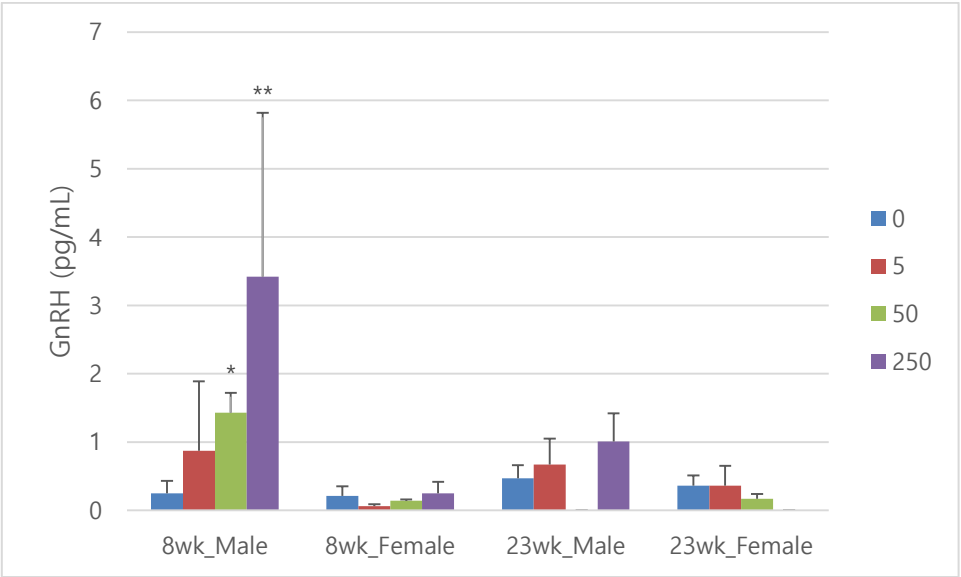
Figure S1 was showed the relative ratio of gonadal fat median (around testis in male, around ovary in female). Also, Figure S2 was showed the median ratio of total fat (visceral adipose tissue, subcutaneous adipose tissue, brown adipose tissue) by sex, time point. Any Differences did not show in median ratio of gonadal fat compared with control group. However, it decreased slightly compared with control group at L group. In total fat, at 8-week-old, the weight of total fat in males was lower than that of the control, but the total fat tended to increase from 8-week-old to 23-week-old. However, in the case of females, the weight of total fat tended to decrease while growing.

3.4 Hormone levels in serum

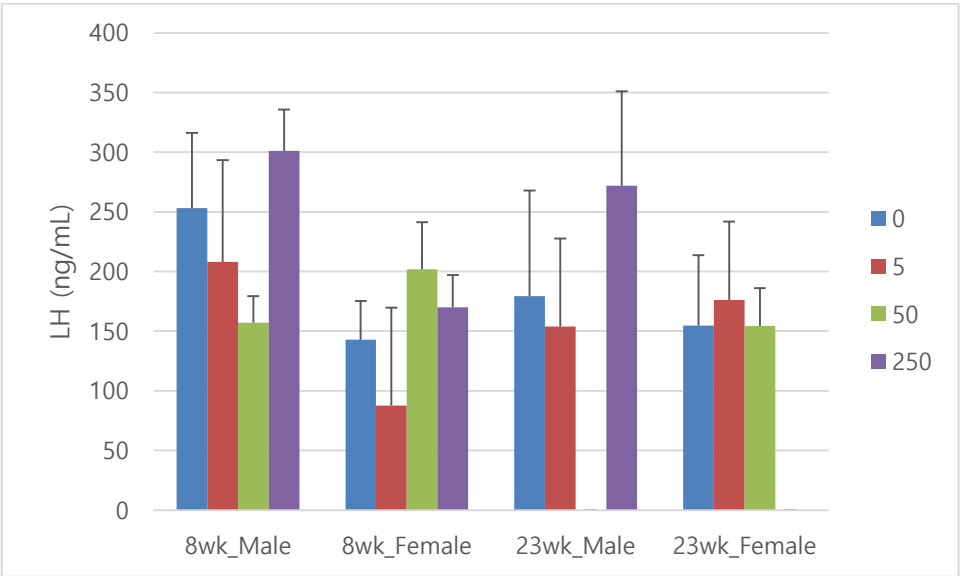
The effects of BPS on hormone levels in the serum related to Hypothalamus-Pituitary-Gonad (HPG) axis of 8weeks old mouse and 23weeks old mouse are shown in Figure 8. The male's GnRH level was higher than female. There was a sex difference in GnRH. Especially in 8 weeks old males, M (50 mg BPS/kg bw /day) group and H (250mg BPS/kg bw/day) group were significant increase compared with control group ($p < 0.05$, $p < 0.01$). In 23 weeks old males, no significant differences were observed in the levels of serum GnRH among the four group, but more than higher in L (5mg BPS/kg bw/day) group and H (250mg BPS/kg bw/day) group compared with control group. Concentration of LH was observed significantly increase in 8 weeks male mouse group than 8 weeks female mouse group. But, no differences was observed by dose of BPS. And there was no sex difference, dose difference at 23 weeks old mouse. However, at H group of all male groups, LH concentration was higher than the control group. Concentration of FSH levels also no significant difference were significantly difference in L group at 8-week-old male mice. Progesterone was not significantly different by time points, but it was higher in females than males. In 8-week-old male mouse's progesterone levels increased in M, H group. Especially, In 8-week-old female mouse's progesterone levels were significantly higher than control group in H group. Testosterone was usually higher in all male groups and sex differences at 8 weeks were significantly prominent. At 8 weeks of age, the concentration of male control group was about 6 times higher than that of female control group. In addition, testosterone levels were significantly higher in the 8 weeks old, M and H group in male than in the control group ($p<0.01$). Also it was significantly higher in the 8 weeks old female mice, H male group than in the control group ($p<0.01$). In estradiol, there was no significant differences in any group. In 8-week-old male L group, estradiol concentration was slightly increased compared to the control group,

but all of the L group except male L group were decreased compared with the control group.

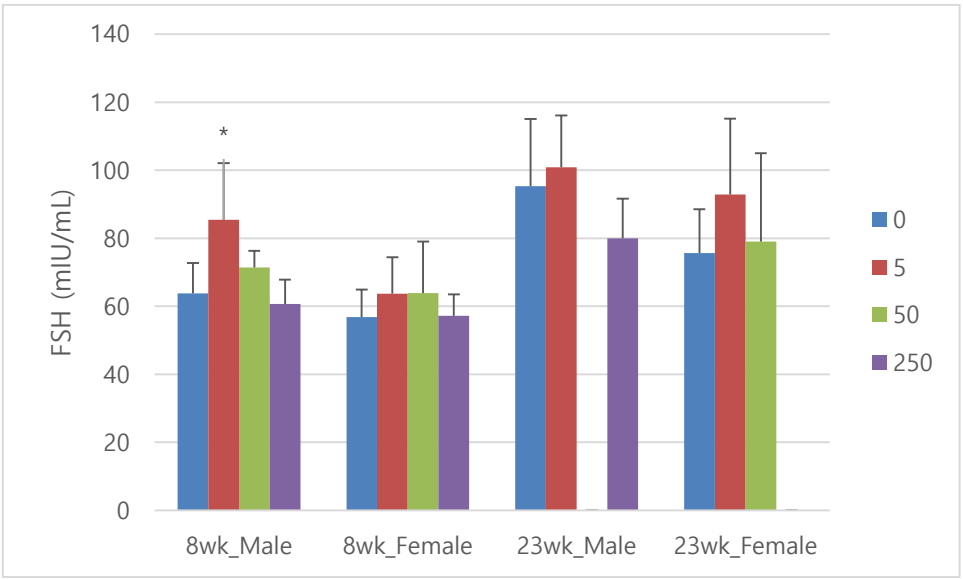
(A) Levels of Gonadotrophin-releasing hormone



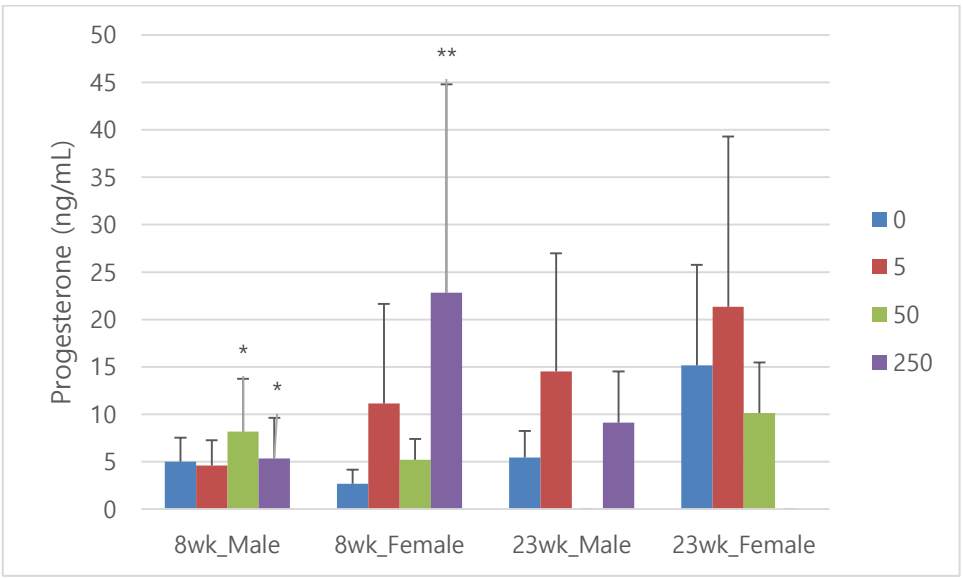
(B) Levels of Luteinizing hormone



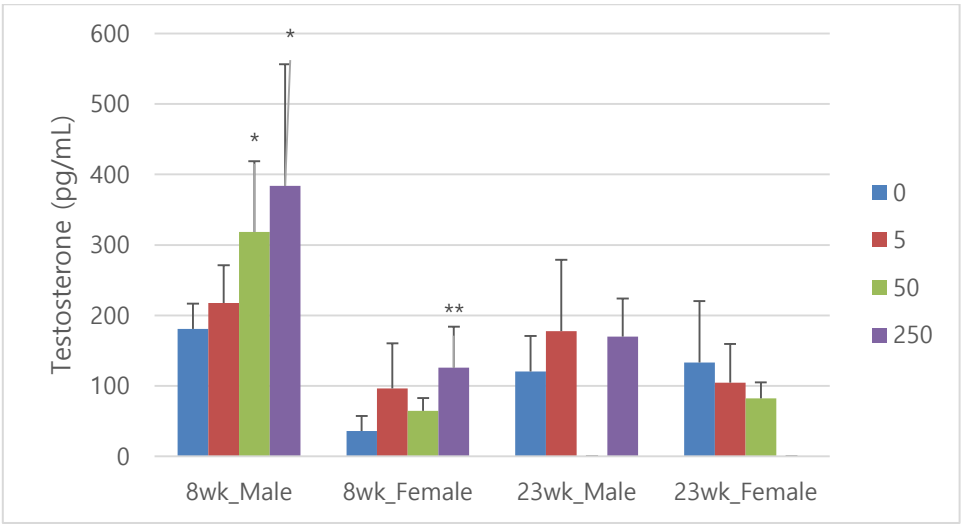
(C) Levels of Follicle stimulate hormone



(D) Levels of Progesterone



(E) Levels of Testosterone



(F) Levels of Estradiol

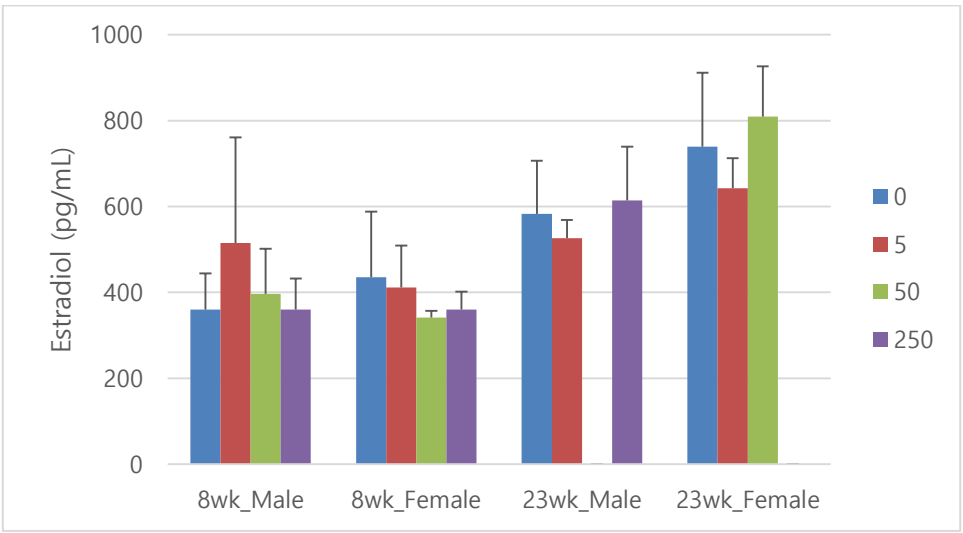


Figure 8. Hormone levels in serum by sex, time point

All value is presented as mean ± standard deviation. GnRH, Gonadotropin-releasing hormone; LH, Luteinizing hormone; FSH, Follicle stimulate hormone. 0, 0mg BPS/kg BW/day (Control); 5, 50mg BPS/kg BW/day; 50, 50mg BPS/kg BW/day; 250, 250mg BPS/kg BW/day;

*p<0.05 significant difference compared to control
**p<0.01 significant difference compared to control

3.3 Identification of metabolites involved in steroidogenesis and metabolism pathways

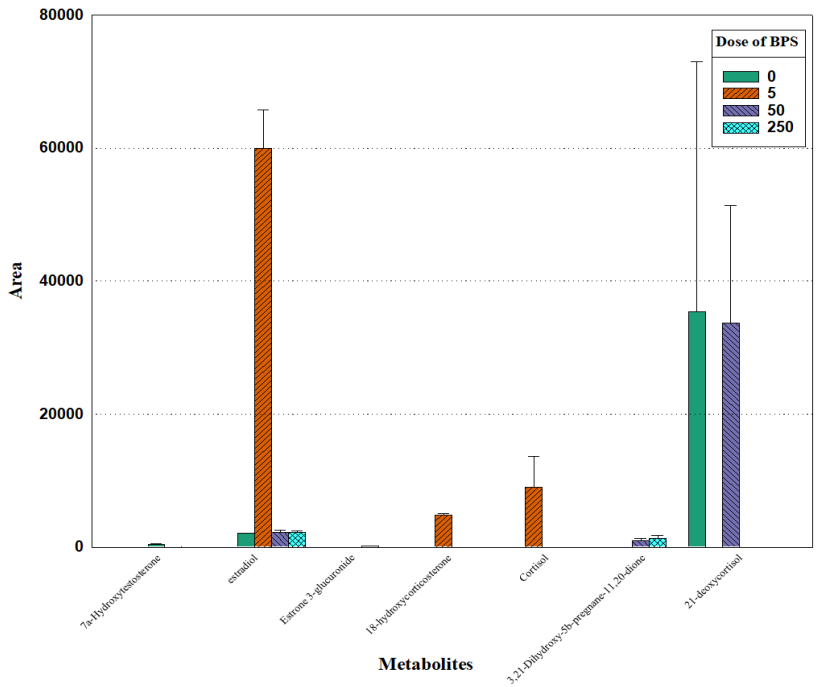
All identification of metabolites of component name, observed *m/z*, mass error, retention time (RT) and adducts were listed in table S1. 13 metabolites have been identified in the androgen group. 10 metabolites have been identified in the estrogen group. In the progestin group, 13 metabolites were founded. And In the corticoids group, 17 metabolites were observed.

Figure 9, 10 showed changes of the steroid metabolism pathway according to sex, according to age. In 8-week-old female, it was difficult to explain the metabolic direction because there were few metabolites detected. Just, estradiol was found to be higher in the group with BPS concentration of 5mg/kg bw/day compared to the other concentration groups. Other than these, hormones in the pathway of synthesis of corticosterone were detected to a small extent. In the exposed group of 8 weeks old male, metabolites contained in the same metabolic pathway were detected in the corticoids, but not in the control group. Especially, 11-Deoxycorticosterone, Corticosterone and 3a,21-Dihydroxy-5b-pregnane-11,20-dione were detected in all treatment group. These metabolites are involved in the pathway in which corticosterone is synthesized and metabolized. The detection rates of these metabolites were in the order of dose of BPS 5, 250 and 50.

In 23 weeks old female, 11-deoxycortisol and 17a,21-Dihydroxy-5b-pregnane-3,11,20-trione of corticoids group were detected at very high. Although the number of metabolites detected to explain the specific pathway was insufficient, both 17a-hydroxyprogesterone and 11b-hydroxyprogesterone of the commonly detected pregestins group were metabolites related in corticoids metabolism pathway. The metabolites were the most abundant in the BPS 5 group, and the control and BPS 50 were similar. Also 16a-Hydroxy-

androstenedione, Androstenedione and Testolactone were detected in BPS 5 group. In 23 weeks old male, 21-deoxycortisol and 17a,21-Dihydroxy-5b-pregnane-3,11,20-trione of corticoids group were detected at very high. However, this is insufficient to explain the metabolic pathway. Especially, the BPS 250 group cannot explain the metabolic pathway because the detected metabolite is rare. However, in the control and BPS 5 groups, metabolites in the direction of metabolism to pregnenolone → progesterone → 11b-hydroxyprogesterone → corticoids group → tetrahydrocorticosterone → 3a, 21-dihydroxy-5b-pregnane-11,20-dione were detected. In addition, these metabolites more in the BPS 5 group than in the control group.

A


Dose of BPS
0
5
50
250
Area
80000
60000
40000
20000
0
7a-Hydroxytestosterone
estradiol
Estrone 3-glucuronide
18-hydroxycortiosterone
Cortisol
3,21-Dihydroxy-5b-pregnane-11,20-diene
21-deoxycortisol
Metabolites

B

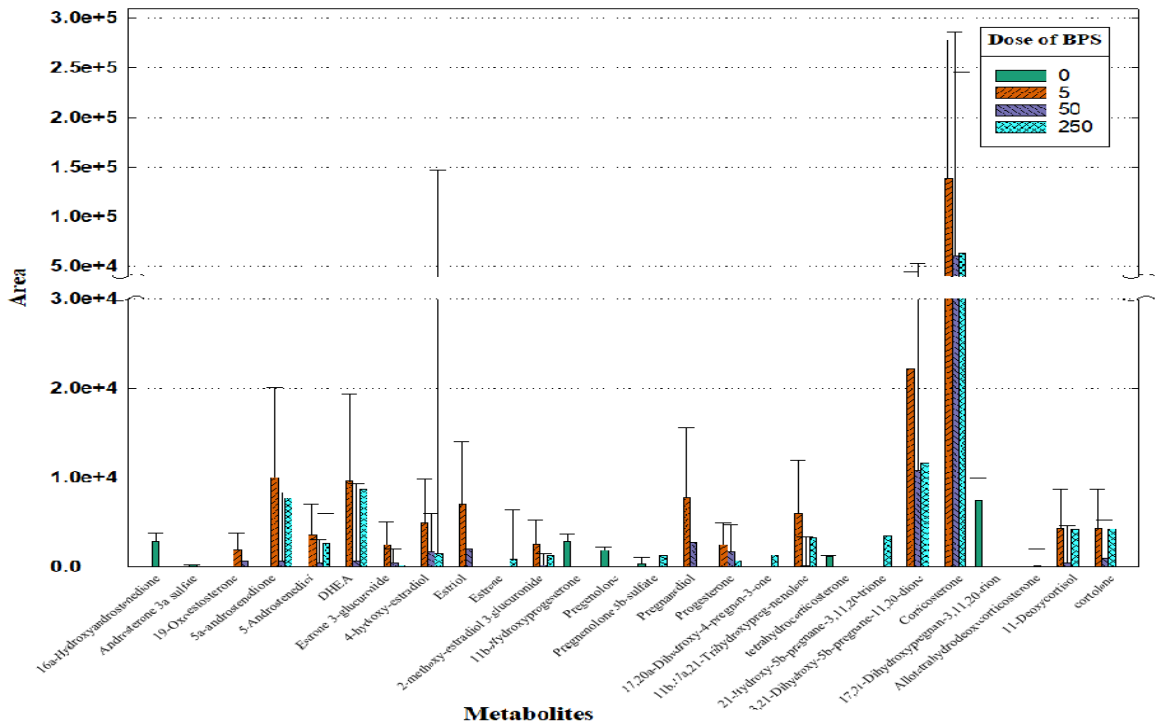
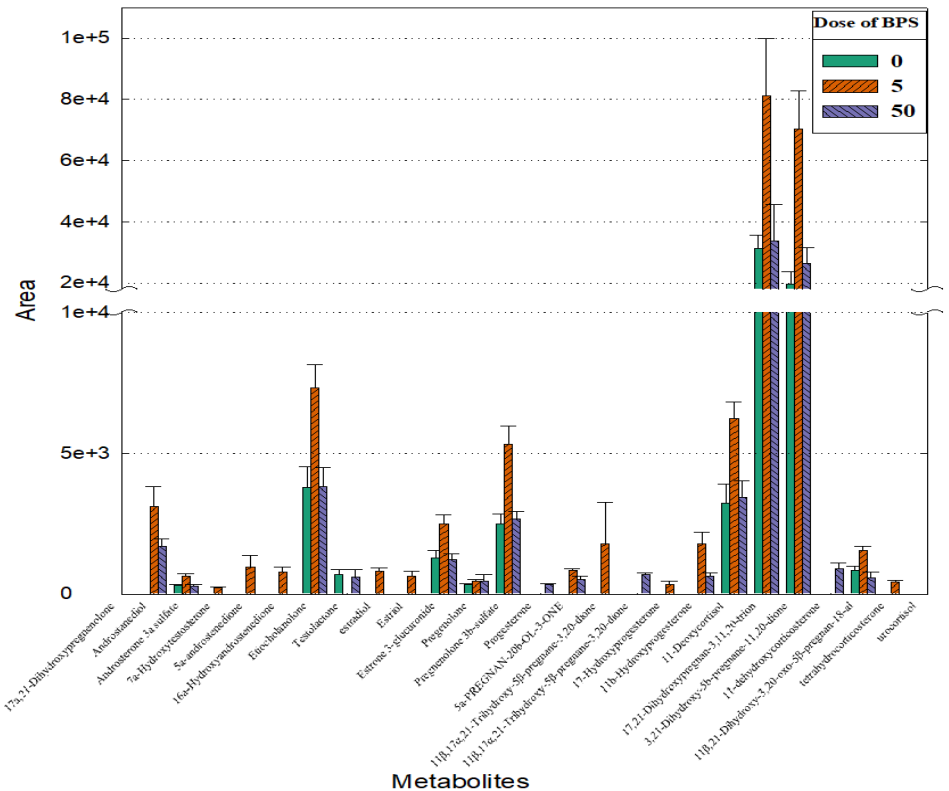


Figure 9. Relative quantification of steroid metabolites of 8weeks old mouse. (A) Female (B) Male.
Each bar and error bar represent area mean and standard deviation of samples.

A


Dose of BPS
0
5
50
Area
1e+5
8e+4
6e+4
4e+4
2e+4
1e+4
5e+3
0
Metabolites
17a,21-Dihydroxypregnenolone
Androstanediol
Androsterone-3a-sulfate
7a-Hydroxytestosterone
5a-androstenedione
16a-Hydroxyandrostenedione
Etiocholanolone
Testolactone
estradiol
Estriol
Estrone-3-glucuronide
Pregnolone
Pregnenolone-3b-sulfate
Progesterone
5a-PREGNAN-20b-OL-3-ONE
11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione
11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione
17-Hydroxyprogesterone
11b-Hydroxyprogesterone
11-Deoxycortisol
17,21-Dihydroxypregnan-3,11,20-trion
3,21-Dihydroxy-5b-pregnane-11,20-dione
11-dehydrocorticosterone
11β,21-Dihydroxy-5,20-oxo-5β-pregnan-18-al
tetrahydrocorticosterone
urocortisol

B

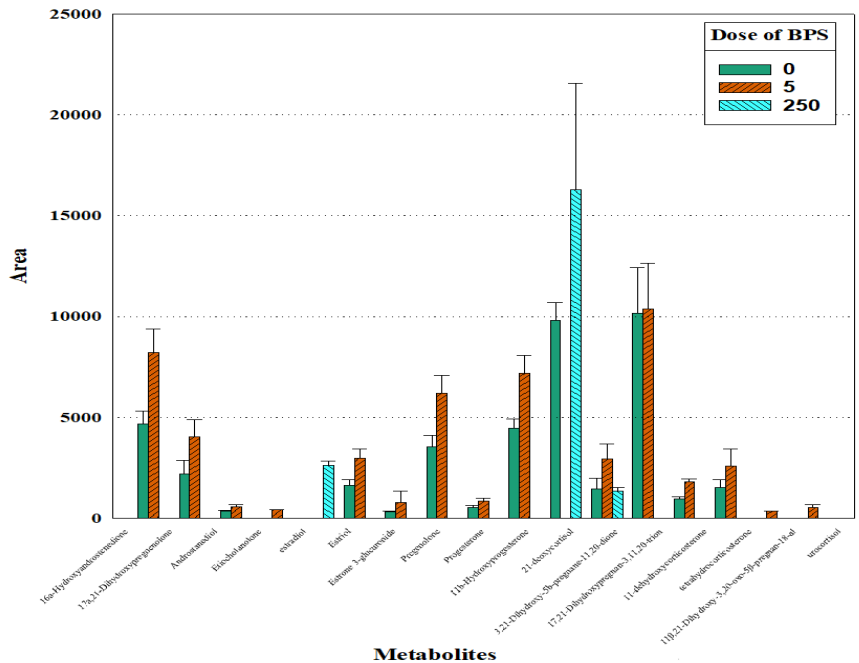


Figure 10. Relative quantification of steroid metabolites of 23 weeks old mouse. (A) Female (B) Male. Each bar and error bar represent area mean and standard deviation of samples.

.IV. Discussion

Recognizing that developing organisms are uniquely sensitive to perturbation by chemicals with estrogenic and/or endocrine disrupting activity, we designed the current study to determine if exposure to BPS during critical developmental windows could disrupt steroid synthesis and metabolism pathway, such that they may respond abnormally to estrogen-like chemicals in later life. In present study, we examined HPG axis related six hormones and steroid hormone metabolites in order to investigate whether prenatal exposure to bisphenol S in mice induced disturbance of steroidogenesis and metabolism pathways.

In the results of physiologic characteristics, it showed that the effects of physiological on prenatal exposure to BPS were significantly different tendency according to the sex, exposure concentration, time point. In particular, kidney and ovary, total fat were different by dose. 5mg/kg bw/day group showed that the weight of the ovary was significantly reduced compared to the control group in 8-week-old group. This observation is expected to be due to the inhibition of follicular growth around puberty by exposure to infants to BPS. However, it is difficult to determine whether the decrease in ovarian weight in 8-week-old is due to the influence of BPS, since the ovarian weight in adult stage is different according to the ovarian follicular developmental stages. Previous research demonstrated that gestational exposure to bisphenol A in mice, ovarian weight at PND 30 was significantly reduced in 1, 10 mg /kg bw/day group, however at PND 70 there was no significant differences among the groups (Nah et al., 2011). But, in another study demonstrated that exposure to bisphenol S has been shown to increase the weight of female utero (Yamasaki et al., 2004). Kidneys showed different tendency according to sex. In females, the relative ratio of the

kidneys was increased according to the dose, while in the male, the relative ratio of the kidneys was decreased only at the concentration of 250mg BPS/kg BW/day at 23-week-old. This can be regarded as a phenotype that bisphenol S disturbs the adrenal steroid hormone or affects kidney toxic such as oxidative damage. In precious study, it has been reported that the administration of 240 mg BPS /kg BW/day to rats increased the weight of kidneys and caused liver and kidney toxicity (Sangai et al.,2014). In males total fat ratio was increased at 8-week-old of 50mg/kg BPS/day. However, At 23 weeks of age, it was similar to the total fat weight of the control group. This causes a change in sex hormones, and prenatal exposure to bisphenol S causes childhood disturbances in adulthood.

Testosterone is essential for germ cell survival and development in the testes. It is synthesized from cholesterol as a substrate in Leydig cells through a series of reactions catalyzed by four enzymes : P450scc, 3β-HSD, CYP17a1 and 17β-HSD (Payne and Hales et al., 2004). P450scc converts cholesterol to pregnenolone, which is the first rate-limiting and hormonally-regulated step in the synthesis of all steroid hormones (Hanukiglu et al., 1992). StAR plays an important role in transferring cholesterol from the outer mitochondrial to the inner mitochondrial membrane in Leydig cells (Manna and Stocco et al., 2005). In the present study, the change in sex steroid hormone levels may cause subsequent steroidogenesis dysfunction by interfering with the regulatory mechanisms of the HPG axis. Another results of our study was that the serum levels of GnRH increased with increasing dose of BPS in 8-week-old males, and the level of testosterone was significantly increased compared with control in 8-week-old females. This observation is consistent with the fact that there are feedback regulatory mechanisms in place to maintain hormonal homeostasis along the HPG-axis. GnRH secretion from the hypothalamus stimulates the pituitary gland to secrete LH, it combined with the LH receptor

of the testis. LH stimulates testicular synthesis and secretion of testosterone from the Leydig cells of males. In this study, there was a negative feedback that LH decreased as testosterone increased in both sexes. This result is different from the previous BPA exposure study. Prenatal exposure to BPA reduced expression levels of GnRH and FSH, also serum T levels in male mice (Wei et al., 2011). Also, low dose BPA exposure decreases pituitary LH secretion, and decreases Leydig cell T production. But, BPA at milligram doses did not affect serum LH and T levels or Leydig cell T production (Benson et al., 2004). Therefore, the differential pattern of decreases observed in the serum levels of LH, T at varying BPA doses implies that dose response of BPA can vary depending on which dose is set because it represents the U-shape. Based on these and other studies, BPS also suggests that the dose response of the U-shape may be expressed.

The limitation of this study is that hypothalamus-pituitary-adrenal (HPA) axis and hypothalamus-pituitary-thyroid (HPT) axis cannot observed. Although the role of estrogenic, androgenic activity of BPS has been demonstrated in vitro, there is no previous study on the role of corticoids, and this study also lacked the adrenal part, so the explanation of the pathway of corticoid synthesis, one of steroidogenesis, was not clear. As a result, many metabolites of corticoids were detected. In both groups of female and male at 23-week-old, were metabolized to pregnolone → tetrahydrocorticosterone pathway according to exposure dose. However, due to the limitations of this study, the corticoids pathway lacked explanation for the steroidogenesis. Another limitation is that the genes involved in steroidogenesis and metabolism pathway are not considered. The results of HPG axis related hormones, their metabolites and physiologic characteristics did not prove that prenatal exposure to BPS causes endocrine disrupting disorders. Therefore further studies additionally are needed to explain better disturbance mechanisms of BPS by observing the

steroidogenesis and metabolism pathway at the gene level.

BPA and substitute of BPA compounds are well-known as weak estrogen agonists (Casals-Casas et al., 2011). Accordingly, we were shown disruption of steroidogenesis and metabolism due to exposure to BPS in rodent. Also, we examined when the disturbance of F1 generation from prenatal exposure to BPS occurred. The present study is the first report to observe the circulation system from synthesis of steroid hormone to metabolic pathway to investigate the effect of exposure to BPS.

V. Conclusions

In conclusion, the present study investigated differences in steroidogenesis and steroid hormones metabolism changes of F1 by sex and time point during the gestation period, exposure to BPS to C57BL/6 pregnant mouse. As a result we were observed disruption of steroidogenesis due to exposure to BPS in rodent. Overall the results of the study showed that the effects of physiological and hormones on prenatal exposure to BPS were significantly different tendency according to the sex, exposure concentration, time point. Also, we examined when the disturbance of F1 generation from prenatal exposure to BPS occurred. Therefore our study could be applied to the study on the mechanism of BPS-induced exposure to the steroid hormone synthesis and metabolism.

VI. References

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VII. Supplementary information

Disruption of the steroidogenesis and steroid metabolism pathway in C57BL/6 mice after prenatal exposure to bisphenol S

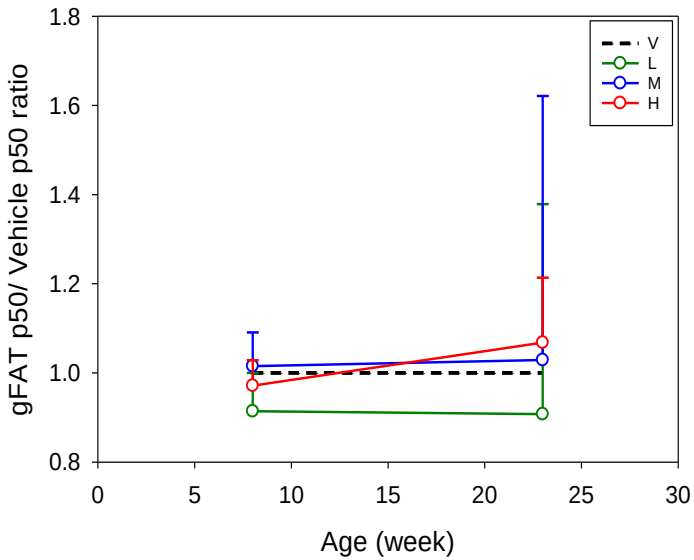
Figure S1. Gonadal fat ratio by sex, time point and dose of BPS.

Figure S2. Total fat ratio by sex, time point and dose of BPS.

Figure S3. Steroidogenesis and steroid metabolism pathways.

Table S1. List of identified metabolites by UPLC-QTOF-MS

(A) Gonadal fat ratio of male



(B) Gonadal fat ratio of female

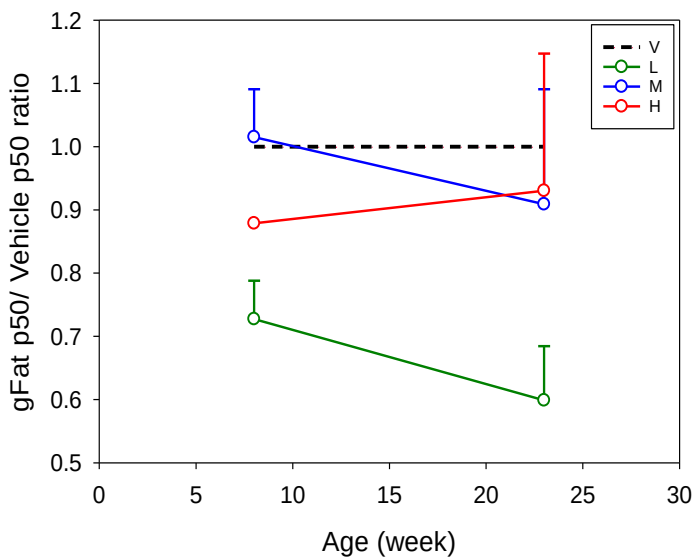
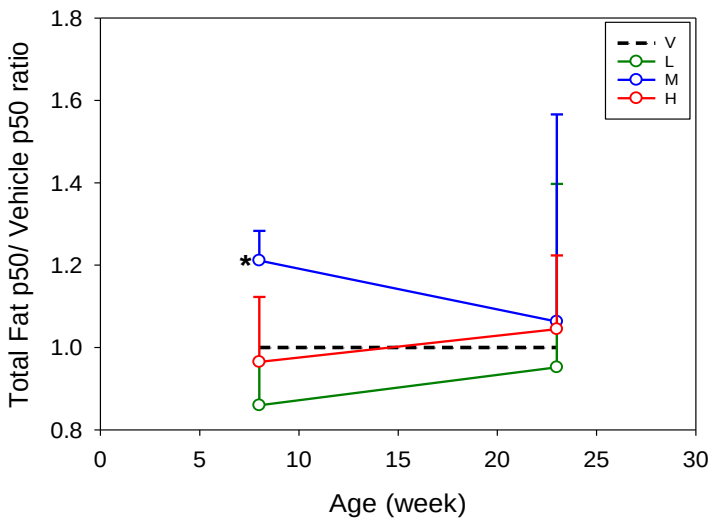


Figure S1. Gonadal fat ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle).

(A) Total fat ratio of male



(B) Total fat ratio of female

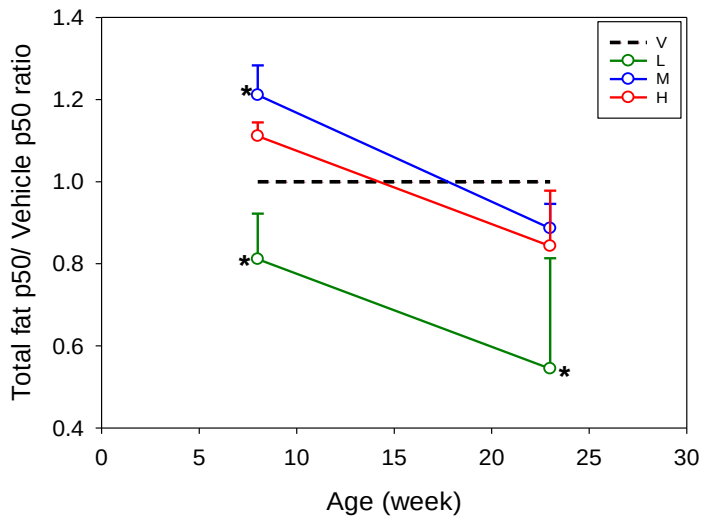
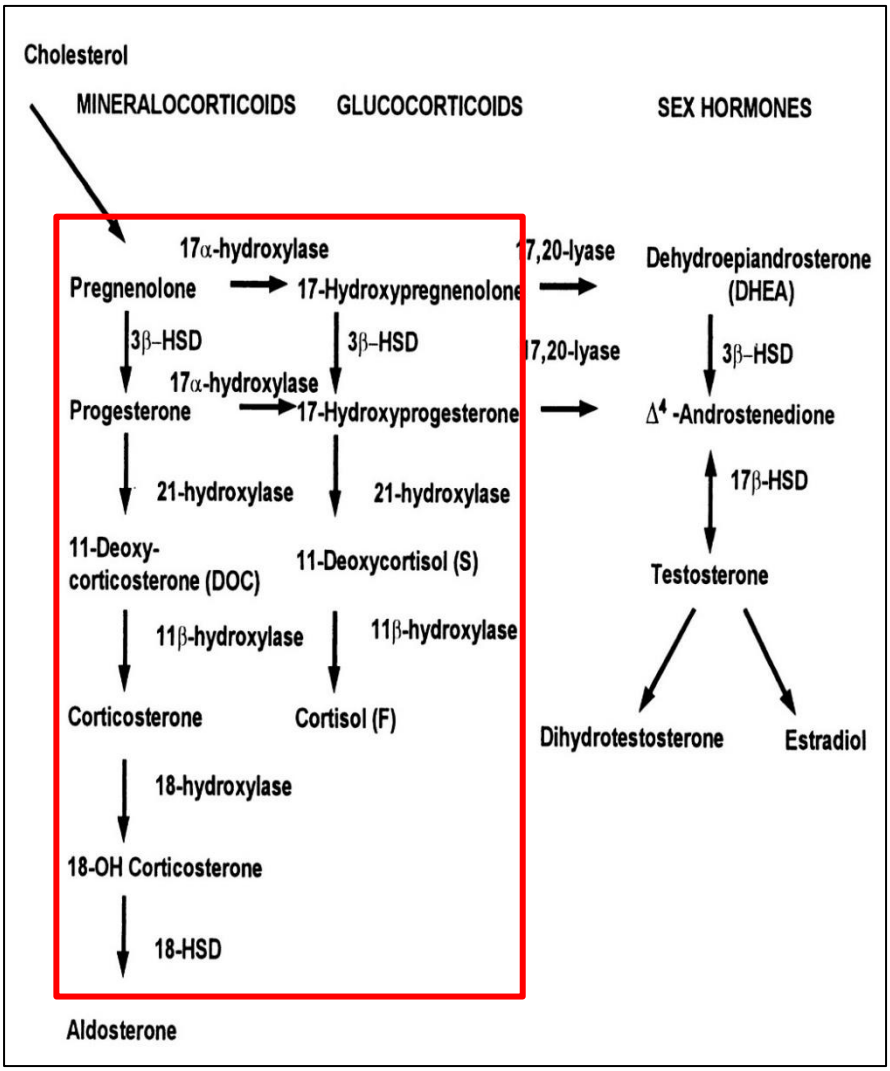


Figure S2. Total fat ratio by sex, time point and dose of BPS

All value is presented as median (p50) / vehicle median (p50) ± standard error (p75).
L = 5mg BPS/kg /day, M = 50mg BPS/kg /day, H = 250mg BPS/kg /day, Total fat
*p<0.05 significant difference compared to control (Vehicle).
**p<0.01 significant difference compared to control (Vehicle)

(A)


Cholesterol
MINERALOCORTICOIDS
GLUCOCORTICOIDS
SEX HORMONES
17α-hydroxylase
Pregnenolone
17-Hydroxypregnenolone
17,20-lyase
Dehydroepiandrosterone (DHEA)
3β–HSD
3β–HSD
17,20-lyase
3β–HSD
17α-hydroxylase
Progesterone
17-Hydroxyprogesterone
Δ4 -Androstenedione
21-hydroxylase
21-hydroxylase
17β-HSD
11-Deoxy-corticosterone (DOC)
11-Deoxycortisol (S)
Testosterone
11β-hydroxylase
11β-hydroxylase
Corticosterone
Cortisol (F)
Dihydrotestosterone
Estradiol
18-hydroxylase
18-OH Corticosterone
18-HSD
Aldosterone

(B)

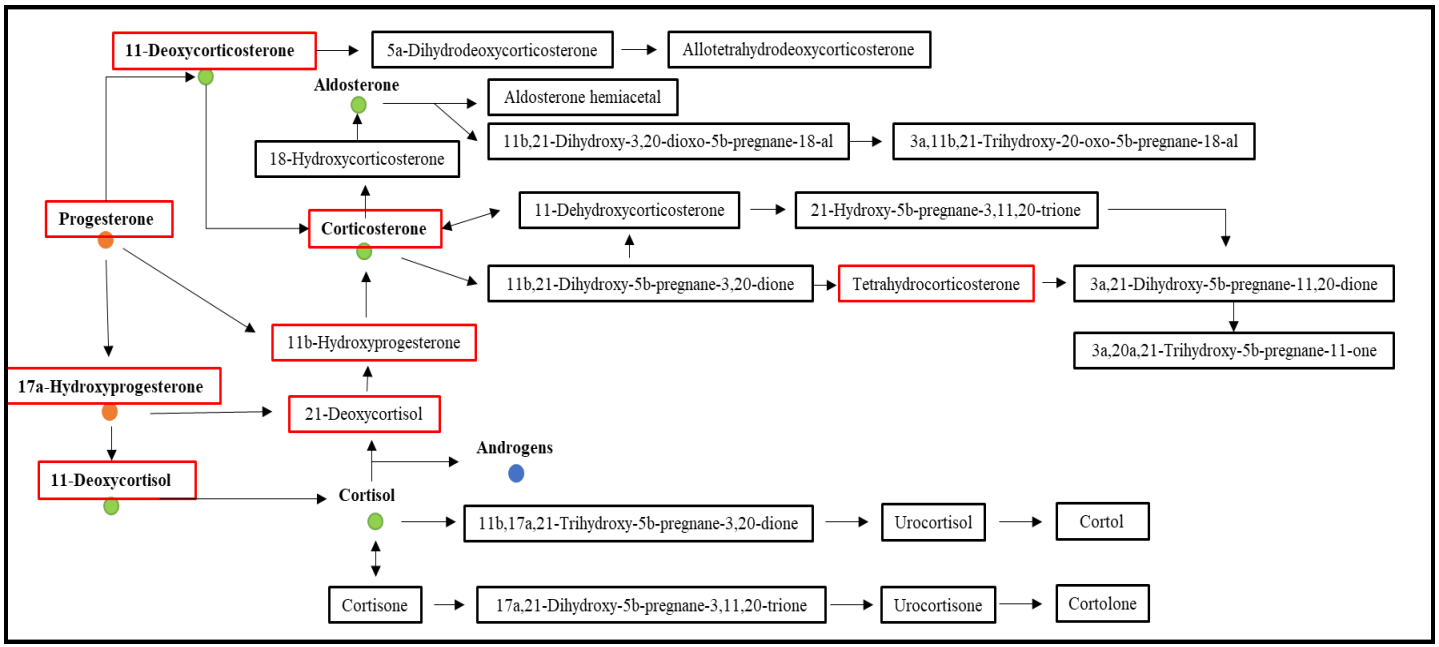


Fig S3. Steroidogenesis and steroid metabolism pathways.
(A) Steroidogenesis pathway (B) Corticoids group metabolism pathway
The red line indicates that the pathway involved in the steroid hormones and metabolites detected in this study
(Adapted from Kegg pathways**;** Jeanneret et al., 2016**)**

Table S1. List of identified metabolites by UPLC-QTOF-MS

No.	Ion Model	Group	Compound name	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts
1	ESI+	Androgens	5a-androstenedione	287.201	0.5	11.38	+H
2	ESI+		19-Oxotestosterone	303.195	-0.5	6.75	+H
3	ESI-		16a-Hydroxyandrostenedione	301.1807	-0.2	11.02	-H
4	ESI-		17a,21-Dihydroxypregnenolone	347.223	0.2	11.49	-H
5	ESI-		Androstanediol	291.234	1	11.26	-H
6	ESI-		Etiocholanolone	289.2178	0.5	10.87	-H
7	ESI-		Testolactone	299.1626	-2.6	11.4	-H
8	ESI-		Etiocholanolone	349.238	-0.5	6.52	+CH3COO
9	ESI-		7a-Hydroxytestosterone	363.2184	0.7	5.51	+CH3COO
10	ESI-		Androsterone-3a-sulfate	429.1944	-0.9	7.56	+CH3COO
11	ESI-		5a-androstenedione	285.1865	0.5	9.62	-H
12	ESI-		5-Androstenediol	289.2174	0.1	9.89	-H
13	ESI-		DHEA	347.2221	-0.7	9.57	+CH3COO
14	ESI+	Estrogens	Estradiol	273.186	1.1	16.95	+H
15	ESI+		Estrone-3-glucuronide	447.2005	-0.9	6.43	+H
16	ESI+		4-hydroxy-estradiol	289.1794	-0.5	16.21	+H
17	ESI+		Estriol	289.1811	1.3	16.64	+H
18	ESI+		Estrone	271.1676	-1.6	9.66	+H
19	ESI+		16a-Hydroxyestrone	287.1635	-0.7	10.98	+H
20	ESI-		Estriol	287.163	-2.3	11.02	-H
21	ESI-		Estrone-3-glucuronide	445.186	-0.8	7.29	-H
22	ESI-		Estradiol	331.1932	1.7	5.67	+CH3COO
23	ESI-		2-methoxy-estradiol-3-glucuronide	477.2138	0.8	7.7	-H

Table S1. Continued

No.	Ion Model	Group	Compound name	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts
25	ESI+		Pregenolone	317.2492	1.7	11.3	+H
26	ESI+		17-Hydroxyprogesterone	331.2237	-3	6.44	+H
27	ESI+		Pregnenolone-3b-sulfate	397.2024	-1.9	13.12	+H
28	ESI+		Pregnanediol	321.2756	-3.2	11.18	+H
29	ESI+		Progesterone	315.234	2.1	7.63	+H
30	ESI+		11b-Hydroxyprogesterone	333.2039	-2.1	6.31	+H
31	ESI-	Progestins	Pregenolone	315.2335	0.5	11.02	-H
32	ESI-		Progesterone	313.2163	-1	11.02	-H
33	ESI-		11b-Hydroxyprogesterone	391.214	1.3	11.04	+CH3COO
34	ESI-		5a-Pregnane-20a-ol-3-one	377.2705	0.7	7.3	+CH3COO
35	ESI-		Pregnenolone-3b-sulfate	455.211	0.1	8.52	+CH3COO
36	ESI-		11b,17a,21-Trihydroxypregnenolone	363.2185	0.8	9.17	-H
37	ESI-		17,20a-Dihydroxy-4-pregnen-3-one	391.2515	2.5	10	+CH3COO

Table S1. Continued

No.	Ion Model	Group	Compound name	Observed *m/z*	Mass error (mDa)	RT (min)	Adducts
38	ESI+		21-deoxycortisol	347.2211	-0.6	5.19	+H
39	ESI+		3,21-Dihydroxy-5b-pregnane-11,20-dione	349.235	0.7	4.66	+H
40	ESI+		Deoxycortisol	347.2205	-1.2	5.36	+H
41	ESI+		11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione	365.2303	-2	3.55	+H
42	ESI+		Corticosterone	347.2201	-1.6	4.89	+H
43	ESI+		21-Hydroxy-5b-pregnane-3,11,20-trione	347.2184	-3.3	4.31	+H
44	ESI+		Allotetrahydrodeoxycorticosterone	335.2573	-0.8	10.87	+H
45	ESI+		17a,21-Dihydroxy-5b-pregnan-3,11,20-trione	363.2178	1.2	8.08	+H
46	ESI+	Corticoids	18-hydroxycorticosterone	363.2161	-0.5	17.48	+H
47	ESI+		Cortisol	363.2149	-1.7	4.75	+H
48	ESI-		11-dehydroxycorticosterone	389.2299	-3.4	11.02	+CH3COO
49	ESI-		Tetrahydrocorticosterone	349.2373	-1.1	12.7	-H
50	ESI-		11β,21-Dihydroxy-3,20-dioxo-5β-pregnan-18-al	421.2262	3	11.68	+CH3COO
51	ESI-		Urocortisol	365.2346	1.2	11.71	-H
52	ESI-		11β,17α,21-Trihydroxy-5β-pregnane-3,20-dione	363.2171	-0.6	5.32	-H
53	ESI-		11-Deoxycortisol	345.2096	2.4	9.33	-H
54	ESI-		Cortolone	365.2305	-2.8	11.07	-H

국문초록

비스페놀 S의 태중 노출로 인한 C57BL/6 마우스 내의 스테로이드 합성 및 대사 경로 교란 연구

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비스페놀 S (BPS)는 비스페놀 A (BPA)의 대체제로 폴리카보네이트 플라스틱, 에폭시 수지 등 다양한 생활용품에 사용되는 물질이다. 국내에서는 BPA의 대체재로 BPS를 주로 사용하고 있으며, 그 사용량이 점차 증가하고 있음에도 불구하고 BPS의 내분비계 교란 작용에 대한 연구는 부족한 실정이다. 따라서 본 연구에서는 BPS의 태중 노출로 인한 F1세 대의 스테로이드 호르몬 합성 및 대사 변화를 관찰하고자 한다. 이를 위해 C57BL/6 임신마우스에게 약 10일 (재태기간 9일 – 출산일) 동안 식수를 통해 BPS를 5mg/kg/day, 50mg/kg/day, 250mg/kg/day로 노출시켰다. 노출된 임신마우스에

게서 태어난 F1세대를 각 8주령, 23주령에 희생하여 신체계측치, 혈청, 지방, 생식기를 포함한 장기를 수집하였다. 수집된 혈청 중 일부는 시상하부-뇌하수체-생식샘 축(HPG axis)에 관여하는 호르몬 6종(생식선자극호르몬방출호르몬(GnRH), 여포자극호르몬(FSH), 황체형성호르몬(LH), 프로게스테론(P4), 테스토스테론(T), 에스트라디올(E2))을 ELISA kit로 측정하였다. 또한, UPLC-qTOF를 이용하여 혈청내의 스테로이드 호르몬의 합성 및 대사과정에 존재하는 모든 스테로이드 호르몬 및 그 대사체들을 측정하였다. 신체계측치의 경우 일반 선형 모델분석 (general linear model analysis) 결과 각 항목은 성별, 주령, 노출 농도에 따라 현저하게 다른 경향성을 나타내었다. 특히, 암, 수 모두 성인기 (23주령) 보다는 사춘기 (8주령)에 유의한 차이를 나타내었다. 시상하부-뇌하수체-생식샘 축에 관여하는 호르몬들의 측정 결과, 8주령 암, 수 마우스 그룹에서 대조군 대비 250mg/kg bw/day 그룹은 GnRH가 높아짐에 따라 LH가 높아지고 T의 수치가 높아지는 경향을 보였다. 스테로이드 호르몬의 합성 및 대사에 관여하는 대사체들은 23주령 암, 수 마우스 그룹과 8주령 수컷마우스의 모든 농도에서 pregenolone으로부터 tetrahydrocorticosterone 방향으로의 대사과정에 두드러진 경향을 보였다. 본 연구는 BPS in vitro 실험 결과와 비교해볼 때, 에스트로겐 활성에 미미한 변화를 일으키는 역할을 한다는 것과 달리 안드로겐 활성을 일으키는 역할을 한다는 결과를 얻었다. 그러나 부신에서의 스테로이드 호르몬 합성과정의 관찰이 부족하여 UPLC-qTOF 측정결과를 설명하기엔 어려움이 있다. 연구 결과, BPS의 태중 노출로 인한 신체계측치 및 호르몬들의 영향은 노출된 BPS의 농도에 따라, 성별에 따라, 관측시기에 따라 확연히 다른 경향성을

나타낸다는 것을 확인하였다. 또한 본 연구는 설치류에서 BPS의 태중 노출로 인한 스테로이드 호르몬 합성 및 대사과정의 순환체계의 변화에 관한 정보를 일부 제공하며 BPS의 독성 기전 연구에 활용될 수 있을 것이다.

주요어 : 비스페놀 S, 태중 노출, HPG axis, 스테로이드 합성 및 대사, 안드로겐 활성

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